REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 18, 2004	Final Progress Report – 12/01/00 – 01/31/04

4. TITLE AND SUBTITLE
Regulation of genes controlling carbohydrate metabolism in the heart of a hibernating mammal

5. FUNDING NUMBERS
DAAD19-01-1-0014

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
41650.7-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)
The project funded by this ARO grant is to characterize genes that are responsible for the physiological changes that occur during hibernation in the heart of the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). We focused on the heart because it is a contractile organ that must continue to work despite physiological conditions that would be lethal to most mammals. We used a PCR-based gene expression screen to isolate cDNAs of genes showing increased levels of expression in the heart during hibernation. In the process we learned a considerable amount about the function of various gene products during hibernation. Specifically, we learned about the genes responsible for the switch from a carbohydrate-based metabolism to a fat-based metabolism and their regulation. We examined novel genomic structures, analyzed the evolution of genes required for hibernation and showed high homology to human orthologs. Finally we demonstrated how fat in the form of triacylglycerols can be hydrolyzed at temperatures as low as 0°C.

14. SUBJECT TERMS	15. NUMBER OF PAGES
hibernation, heart, genes, low-temperature, metabolism	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Final progress report for U.S. Army Research Office grant -"Regulation of genes controlling carbohydrate metabolism in the heart of a hibernating mammal" DAAD19-01-1-0014

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I. FORWARD

A growing number of advances in both molecular and bioinformatic technologies have recently allowed systematic investigations of previously intractable biological systems. So-called "nontraditional" species with unique physiological properties are now on the brink of being explored at a level of detail only previously realized with certain model organisms. Mammalian hibernation is one example of a complex physiological process that is now poised for such an investigation. Hibernating mammals provide a unique system for identifying molecules that are important in regulating cardiac function under suboptimal conditions of body temperature, oxygen consumption and fuel supply. In a state of deep hibernation, body temperature hovers a few degrees above 0°C for periods of months, oxygen consumption holds at 1/30 to 1/50 of the aroused condition and heart rate can be as low as 3-10 beats/minute, compared to 200-300 beats/minute when the animal is awake and active (reviewed in [1]).

The project funded by this ARO grant was to characterize genes that are responsible for the physiological changes that occur during hibernation in the heart of the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). We focused on the heart because it is a contractile organ that must continue to work despite physiological conditions that would be lethal to most mammals. We used a PCR-based gene expression screen to isolate cDNAs of genes showing increased levels of expression in the heart during hibernation [2]. In the process we learned a considerable amount about the function of various gene products during hibernation. Specifically, we learned about the genes responsible for the switch from a carbohydrate-based metabolism to a fat-based metabolism, and how fat in the form of triacylglycerols can be hydrolyzed at temperatures as low as 0°C.

II. PROBLEM STUDIED

We monitored changes in mRNA levels to identify molecules that are important for maintaining heart function under extreme physiological conditions. This approach offers an important and under exploited opportunity to gain information about the molecular events that allow hibernators to control and survive the hibernating state. Evolutionary arguments indicate that genes required for induction and maintenance of

the hibernating state are not likely to be unique to mammals that hibernate. Thus, by identifying differentially expressed genes, and their role in the physiology of hibernation, we expect to apply this knowledge to help the warfighter become more tolerant of hypothermia and ischemia.

The high homology of *Spermophilus tridecemlineatus* genes with their respective human and rodent orthologs is shown in Table 1 for four cDNAs that we previously cloned and characterized. This analysis shows the high probability of identifying ground squirrel gene products by comparison with well-characterized mammalian genomes.

***S. tridecemlineatus* cDNA (with Accession nos.)**	**Percent Nucleotide Identity**		
	Human	Mouse	Rat
Hormone Sensitive Lipase AF177401	88.2	87.9	85.1
Pyruvate Dehydrogenase Kinase-4 AF020845	88.8	86.2	85.8
Pancreatic Triacylglycerol Lipase AF02793	86.1	n.a.	83.4
26S proteasome- sug2 U36395	94.0	n.a.	n.a.
TABLE 1.	n.a.-sequence not available		

We have identified a set of metabolic genes responsible for the physiological characteristics seen in the heart during hibernation. We found that genes encoding pancreatic triacylglycerol lipase (PTL) and pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) are up-regulated in the heart when hibernation begins, and that steady-state levels of both mRNAs remain high while metabolism and body temperature are greatly depressed [2]. This functional genomics approach has allowed us to construct and test model biochemical pathways specific to the heart of the hibernating animal (Figure 1).

PDK4 prevents the flow of glycolytic intermediates into the tricarboxylic acid (TCA) cycle and therefore spares glucose and limits carbohydrate oxidation. PTL hydrolyzes triacylglycerols to liberate free fatty acids to be used as fuel. Involvement of PDK4 and PTL in the intermediary metabolism of the ground squirrel heart contributes to the observed respiratory quotient (RQ) of 0.7 seen during hibernation. RQ is a unit-less value representing the moles of CO_2 respired per moles of O_2 consumed. A value of 1.0 indicates combustion of carbohydrates; however, an RQ of 0.7 indicates that fat is the major substrate for energy production.

III. SUMMARY OF MOST IMPORTANT RESULTS

Novel expression of PTL. Until recently, PTL was thought to be expressed exclusively in the pancreatic acinar cells and secreted into the small intestine as a means to digest dietary fat. However, PTL mRNA and enzymatic activity was unexpectedly found in the hibernating heart [2]. Since that initial finding we have found PTL expression during hibernation in other novel locations such as white adipose tissue

and testis [3]. Extracts from ground squirrel hearts showed that PTL retains high lipolytic activity at extremely low temperatures [2]. To determine whether this cold lipolysis is unique to ground squirrel PTL, or possibly the result of a hibernation-specific modification of the lipase, we expressed cDNAs for both human and thirteen-lined ground squirrel PTL in the yeast *Pichia pastoris* [4].

Human enzymatic activity at 0° C. We found that both human and ground squirrel PTL perform remarkably well at temperatures as low as 0°C. We conclude that low-temperature lipolysis is a property of both human and thirteen-lined ground squirrel PTL, and that PTL does not require modifications specific to mammalian cells in order to function in the cold. More importantly it demonstrates that hibernators use genes common to all mammals for purposes of surviving stasis and shows that the low-temperature catalysis seen with a protein in hibernators is also a characteristic of the same enzyme found in humans.

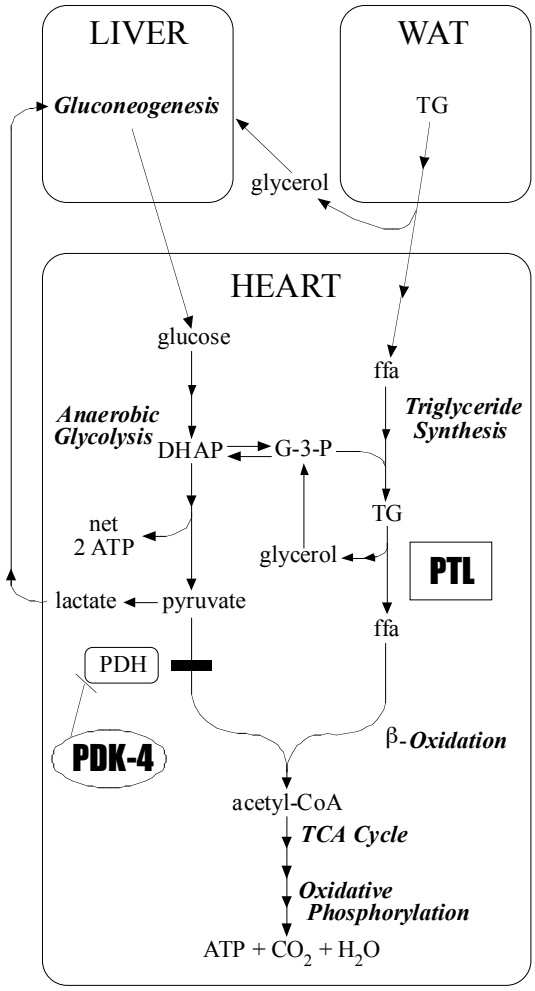

Figure 1. Model showing the metabolic involvement of pyruvate dehydrogenase kinase isozyme 4 (PDK-4) and pancreatic triacylglycerol (PTL) lipase in the heart of a hibernating thirteen-lined ground squirrel. Names of metabolic pathways are shown in Italics. Arrows with a single arrowhead indicate a single reaction. Continuous arrows with two or more arrowheads indicate multi-step pathways. Abbreviations: ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; ffa, free fatty acid; G-3-P, L-glycerol 3-phosphate; PDH, pyruvate dehydrogenase; TCA cycle, tricarboxylic acid cycle; TG, triacylglycerols; WAT, white adipose tissue. Figure modified from Andrews et al. [2].

Induction of hibernation-associated gene activity by circulating effector molecules. Identification of specific genes associated with the hibernating state allows researchers to identify and test endogenous and exogenous factors that control differential gene expression during hibernation. Coordinate activation of the gene encoding PDK4 in different tissues throughout the body points to circulating effector molecules that regulate its expression during hibernation [5]. Figure 2 is a model showing changes in serum insulin and fatty acid levels regulate PDK4 expression and ultimately the switch from carbohydrate- to fat-based catabolism during hibernation. Insulin has been shown to repress PDK4 gene activity, while specific fatty acids act as

ligands for peroxisome proliferator activated receptor alpha (PPARα), which activates the PDK4 gene in a ligand dependent manner.

These observations demonstrate the importance of identifying genes that are differentially regulated during hibernation. Changes in the levels of gene products that control the hibernating phenotype can be used to identify potential pharmacological targets. By gaining a fuller understanding of the molecular mechanisms that control hibernation, we hope to develop pharmacological strategies for duplicating the cardioprotective properties of hibernation in humans.

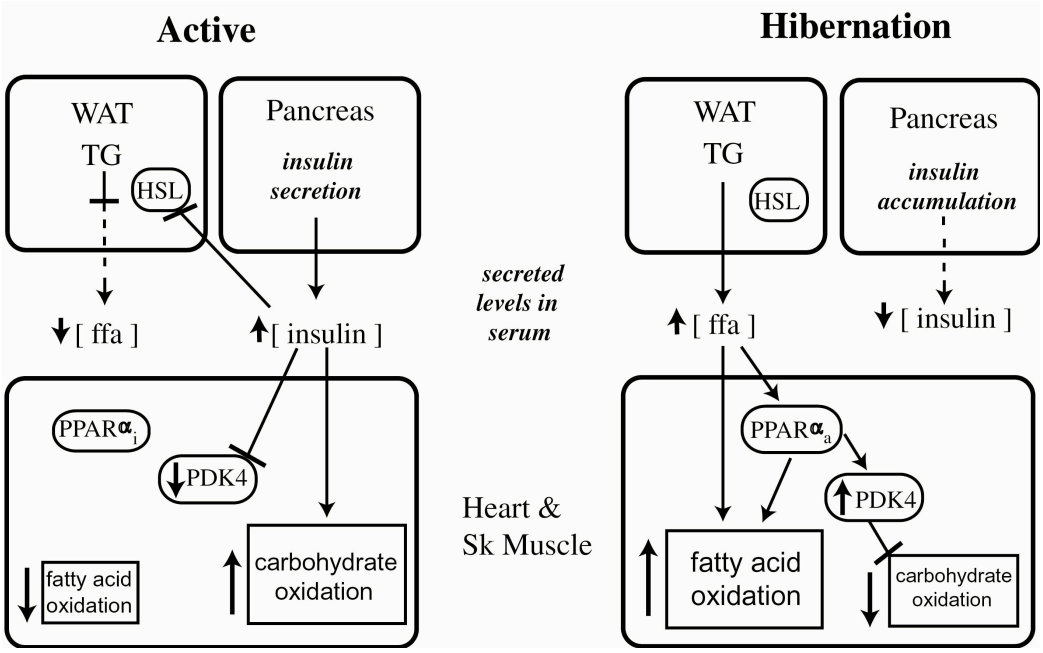

Figure 2. Model showing regulation of the switch from carbohydrate to fatty acids as the primary source of fuel during hibernation. Effects of serum levels of insulin secreted from the pancreas and free fatty acids secreted from WAT on PDK4 gene expression, carbohydrate oxidation and fatty acid oxidation in heart and skeletal muscle in active animals that are fattening (Active; September-October) and in animals that are hibernating (Hibernation; December-January). Lines with arrowheads indicate up-regulation or activation and lines with blunt ends indicate down-regulation or inhibition. Solid lines show the active or predominant mode of regulation and dashed lines show minor pathways. Abbreviations: ffa, free fatty acids; HSL, hormone sensitive lipase; PPARα, peroxisome proliferator activated receptor alpha; $PPAR\alpha_i$, inactive receptor; $PPAR\alpha_a$, active receptor; PDK4, pyruvate dehydrogenase kinase isoenzyme 4; TG, triacylglycerols; WAT, white adipose tissue.

Identification of hibernation-related pharmacological targets. PDK4 expression is induced in skeletal muscle of rats and the hearts of mice by a hypolipidemic drug and known peroxisome proliferator, WY-14,643 [6,7]. This finding indicates a role for peroxisome proliferator-activated receptor alpha (PPARα) in controlling PDK4 gene expression due to the fact that WY14,643 (shown in Figure 3) is a selective PPARα-activating ligand [8]. The action of WY-14,643 is an example where pharmacological intervention leads to gene expression patterns characteristic of hibernation.

The role of PPARα in activating the PDK4 gene has also been confirmed using PPARα-null mice [7]. Of key importance to hibernation is the potential role of PPARα in coordinating PDK4 gene expression with the expression of genes responsible for extracellular and intracellular lipid transport and mitochondrial β-oxidation of fatty acids [9]. As shown in Figure 2, activation of PPARα provides a mechanism linking the inhibition of carbohydrate oxidation with increased fatty acid oxidation, thus accounting for the switch in fuel selection seen during hibernation.

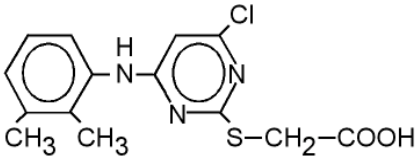

Figure 3. Structure of the hypolipidemic drug WY-14,643 which has been shown to selectively activate PPARα and increase expression of genes involved in the switch from carbohydrate-based to fat-based metabolism.

IV. SPECIFIC FINDINGS

Expression of a chimeric retroviral-lipase mRNA confers enhanced lipolysis in a hibernating mammal. Hibernating mammals can survive several months without feeding by limiting their carbohydrate catabolism and using triacylglycerols stored in white adipose tissue (WAT) as their primary source of fuel. In this project we showed that a lipolytic enzyme normally found in the gut, pancreatic triacylglycerol lipase (PTL), is expressed in WAT of hibernating 13-lined ground squirrels (*Spermophilus tridecemlineatus*). PTL expressed in WAT is encoded by an unusual chimeric retroviral-PTL mRNA approximately 500 bases longer than the predominant PTL message found in other ground squirrel tissues. Seasonal measurements detect the chimeric mRNA and PTL enzymatic activity in WAT before and during hibernation, with both showing their lowest observed levels 1 week after hibernation concludes in mid-March. PTL is expressed in addition to hormone-sensitive lipase, the enzyme typically responsible for hydrolysis of triacylglycerols in WAT. Because of the distinct catalytic and regulatory properties of both enzymes, this dual-triacylglycerol lipase system provides a means by which the fuel requirements of hibernating 13-lined ground squirrels can be met without interruption.

Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. Hibernation in mammals requires a metabolic shift away from the oxidation of carbohydrates and toward the combustion of stored fatty acids as the primary source of energy during torpor. A key element involved in this fuel selection is pyruvate dehydrogenase kinase isoenzyme 4 (PDK4). PDK4 inhibits pyruvate dehydrogenase and thus minimizes carbohydrate oxidation by preventing the flow of glycolytic products into the tricarboxylic acid cycle. This project examined expression of the PDK4 gene during hibernation in heart, skeletal muscle, and white adipose tissue

(WAT) of the 13-lined ground squirrel, *Spermophilus tridecemlineatus*. During hibernation PDK4 mRNA levels increase 5-fold in skeletal muscle and 15-fold in WAT compared with summer-active levels. Similarly, PDK4 protein is increased threefold in heart, fivefold in skeletal muscle, and eightfold in WAT. High levels of serum insulin, likely to have an inhibitory effect on PDK4 gene expression, are seen during fall when PDK4 mRNA levels are low. Coordinate upregulation of PDK4 in three distinct tissues suggests a common signal that regulates PDK4 expression and fuel selection during hibernation.

Novel genomic organization of pancreatic triacylglycerol lipase in a hibernating mammal. Pancreatic triacylglycerol lipase (*PTL*) is expressed in novel locations during hibernation in the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). PTL cDNAs isolated from two of these locations, heart and white adipose tissue (WAT), contain divergent 5'-untranslated regions (5'-UTRs) suggesting alternative promoter usage or the possibility of multiple *PTL* genes in the ground squirrel genome. In addition, cDNAs isolated from WAT contain tracts of retroviral sequence in their 5'-UTRs. Our examination of PTL genomic clones isolated from a thirteen-lined ground squirrel genomic DNA library, coupled with genomic Southern blot analysis, enabled us to conclude that PTL mRNAs expressed in heart and WAT are the products of the same single copy gene. The 5'-portion of this gene spans 9.2 kb, is composed of 6 exons, and contains a full-length endogenous retroviral genome with conserved long terminal repeats (LTRs). Alignment of the ground squirrel *PTL* gene with the mouse, rat, and human *PTL* genes indicates that this retrovirus inserted into the ground squirrel genome approximately 200 bases upstream of the *PTL* transcriptional start site. The insertion is a relatively recent event based on largely intact open reading frames containing minimal frameshift and nonsense mutations. The high percentage identity (99.2%) shared between the 5' and 3' LTRs of this endogenous retrovirus suggests that the insertion occurred as recently as 300,000 years ago.

Cold-adapted function and differential expression of pancreatic triacylglycerol lipase in a hibernating mammal. Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) exploit the low-temperature activity of pancreatic triacylglycerol lipase (PTL) during hibernation. Lipolytic activity at body temperatures associated with hibernation was examined using recombinant ground squirrel and human PTLs expressed in yeast. Both the human and ground squirrel enzymes displayed high activity at temperatures as low as 0°C and showed Q_{10} values of 1.2-1.5 over a range of 37 to 7°C. These studies indicate that low-temperature lipolysis is a general property of PTL and does not require protein modifications unique to mammalian cells and/or the hibernating state. Western blots show elevated levels of PTL protein during hibernation in both heart and white adipose tissue (WAT). Significant increases in PTL gene expression are seen in heart, WAT and testis; but not in pancreas where PTL mRNA levels are highest. Up-regulation of PTL in testis is also accompanied by expression of the PTL-specific cofactor, colipase. The multi-tissue expression of PTL during hibernation supports its role as a key enzyme that shows high activity at low temperatures.

Steroidogenesis and the HPA Axis during Hibernation: Differential Expression of the StAR Protein. This project examined a rate-limiting component of the hypothalamic-pituitary-adrenal (HPA) axis during mammalian hibernation. Specifically, we analyzed the seasonal expression of the Steroidogenic Acute Regulatory protein (StAR) in the adrenals of the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*. StAR activity is a rate-limiting step in steroidogenesis and is required for production of a class of steroid hormones that regulate metabolism called glucocorticoids. In ground squirrels, physiologically important glucocorticoids include cortisol, and to a lesser extent, corticosterone. We have hypothesized that regulation of adrenal steroidogenesis during hibernation is mediated by changes in the concentration of StAR protein. We found that levels of StAR mRNA and protein decline during the autumn months, and are significantly reduced during deep hibernation when animals show depressed metabolism and body temperatures of 4-6°C. These results suggest that the StAR protein may play an important role in regulating the hibernation phenotype.

V. PUBLICATIONS Resulting from grant

a.) Papers published in peer reviewed journals

Bauer, V.W., Squire, T.L., Lowe, M.E., and Andrews, M.T. (2001) Expression of a chimeric retroviral-lipase mRNA confers enhanced lipolysis in a hibernating mammal. *Am. J. Physiol.* 281, R1186-1192.

Buck, M.J., Squire, T.L., and Andrews, M.T. (2002) Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. *Physiol. Genomics* 8: 5-13.

Carey, H.V., Andrews, M.T, and Martin, S.L., (2003) Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews*, 83, 1153-1181.

Squire, T.L. and Andrews, M.T. (2003) Pancreatic triacylglycerol lipase in a hibernating mammal: I. Novel genomic organization. *Physiol. Genomics* 16: 119-130.

Squire, T.L., Bauer, V.W., Lowe, M.E., and Andrews, M.T. (2003) Pancreatic triacylglycerol lipase in a hibernating mammal: II. Cold-adapted function and differential expression. *Physiol. Genomics* 16: 131-140.

b.) Papers published in non-peer-reviewed journals or in conference proceedings

Andrews, M.T., Tredrea, M.M. and Shaw, A.K. (2004) Steroidogenesis and the HPA Axis during Hibernation: Differential Expression of the StAR Protein. in *"Life in the Cold"* (B. Barnes and H. Carey, Eds.) in press.

VI. PARTICIPATING SCIENTIFIC PERSONNEL

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Teresa L. Squire, graduate student, Ph.D. received 2002.
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VII. INVENTIONS

None

VIII. BIBLIOGRAPHY

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